Early chronic ethanol exposure in rats disturbs respiratory network activity and increases sensitivity to ethanol

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Chronic ethanol exposure during the fetal period alters spontaneous neuronal discharge, excitatory and inhibitory amino acid neurotransmission and neuronal sensitivity to ethanol in the adult brain. However, nothing is known about the effects of such exposure on the central respiratory rhythmic network, which is highly dependent on ethanol-sensitive amino acid neurotransmission. In 3- to 4-week-old rats, we investigated (1) the effects of chronic ethanol exposure (10% v/v as only source of fluid) during gestation and lactation on phrenic (Phr) and hypoglossal (XII) nerve activity using an *in situ* preparation and on spontaneous breathing at rest in unanaesthetized animals using plethysmography; (2) the sensitivity of the respiratory system to ethanol re-exposure *in situ*; and (3) the phrenic nerve response to muscimol, a $GABA_A$ receptor agonist, applied systemically in an *in situ* preparation. In control rats, ethanol (10–80 mM) induced a concentration-dependent decrease in the amplitude of both XII and Phr motor outflows. At 80 mM ethanol, the amplitude of the activity of the two nerves displayed a difference in sensitivity to ethanol and respiratory frequency increased as a result of shortening of postinspiratory duration period. After chronic ethanol exposure, respiratory frequency was significantly reduced by 43% *in situ* and by 23% in unanaesthetized animals, as a result of a selective increase in expiratory duration. During Phr burst, the ramp was steeper, revealing modification of inspiratory patterning. Interestingly that re-exposure to ethanol *in situ* elicited a dramatic inhibitory effect. At 80 mM, ethanol abolished rhythmic XII nerve outflow in all cases and Phr nerve outflow in only 50% of cases. Furthermore, administration of 50 μM muscimol abolished Phr nerve activity in all control rats, but only in 50% of ethanol-exposed animals. Our results demonstrate that chronic ethanol exposure at an early stage of brain development depresses breathing in juvenile rats, and sensitizes the respiratory network to re-exposure to ethanol, which does not seem to involve GABAergic neurotransmission.

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Neurophysiological mechanisms involved in the effects of ethanol during brain development are not fully understood at the present time. In humans, prenatal ethanol exposure can induce fetal alcohol syndrome characterized by mental retardation not compensated by further development (Burd *et al.* 2003). Studies characterizing the consequences of prenatal ethanol exposure generally describe an aberrant neurophysiology in young or adult offspring (Richardson *et al.* 2002; Iqbal *et al.* 2004). These abnormalities may result from a maladaptive alteration of the brain neuronal network underlying complex physiological functions. Alterations may include developmental delay in neuronal network organization and/or changes in neuronal properties that may influence overall network activity. Considerable research indicates that the major action of ethanol is via $GABA_A$ and NMDA receptors (Faingold *et al.* 1998), resulting in neuronal apoptotic processes during developmental periods (Ikonomidou *et al.* 2000). Other consequences of prenatal ethanol exposure include a decrease in the number of spontaneous active neurons in some adult rat brain areas (Choong & Shen, 2004) or potentiation of $GABA_A$-mediated current modulation (Allan *et al.* 1998), while NMDA receptor activity is probably lower (Costa *et al.* 2000*b*). Despite these reports, nothing is known about the consequences of early chronic ethanol exposure on the activity of central autonomous rhythmic networks underlying physiological functions such as the cardio-respiratory networks embedded within the brainstem. However, acute ethanol exposure is known

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to strongly depress respiratory related hypoglossal and phrenic nerve output in humans and other mammals, both *in vivo* and *in vitro* via NMDA, glycine and $GABA_A$ receptors (Krol *et al.* 1984; St John *et al.* 1986; Haji & Takeda, 1987; Di Pasquale *et al.* 1995; Gibson & Berger, 2000). We hypothesized that early chronic ethanol exposure during the developmental period of the rat brain disturbs the central rhythmic network that controls breathing. Our assumption was that the network produces a different rhythm because ethanol could affect the main neurotransmitter systems underlying this activity (i.e. NMDA and glycine/GABA receptors). We also tested the sensitization or tolerance of rhythmic activity in response to re-exposure of ethanol after early chronic ethanol exposure and the possibility that enhancement of endogenous GABA neurotransmission was responsible for the observed changes. In addition, we recorded breathing at rest by plethysmography to reveal alterations in intact animals. This study may help to understand the changes induced in central neuronal network activity after early chronic exposure to ethanol.

Methods

Animals and surgery

Experiments were performed on 41 juvenile Sprague-Dawley rats (3–4 weeks old) of either sex. Control animals were offspring from female rats that had not been exposed to ethanol (nine different litters). For the ethanol-exposed group of animals, female rats received a 10% (v/v) ethanol solution as their only drinking fluid for 4 weeks before mating and throughout the gestation and lactation periods (Naassila & Daoust, 2002). Offspring from seven different litters formed the ethanol-exposed animal group for *in situ* and *in vivo* experiments. Food was provided *ad libitum* in the two groups of female rats.

The procedures described for surgery are in accordance with the guidelines for care and use of laboratory animals adopted by the European Community, law 86/609/EEC. *In situ* working heart–brainstem preparations were performed as previously described (Leiter & St-John, 2004). Under deep halothane anaesthesia, as assessed by lack of withdrawal reflex to nociceptive paw pinch, the animal was decerebrated at the precollicular level and the forebrain was removed by aspiration. The animal was then transected below the diaphragm, and the phrenic and hypoglossal nerves were isolated and cut distally before placing the animal in the recording chamber. The descending aorta was perfused with an artificial cerebrospinal fluid (aCSF) containing (mM): $MgSO_4$ 1.25, KH_2PO_4 1.25, KCl 5, $NaHCO_3$ 25, NaCl 125, glucose 10 and $CaCl_2$ 2.5, with 1.25% ficoll[70], maintained at 31°C, equilibrated and continuously gassed with carbogen (95% O_2–5% CO_2). Animals were then paralysed by pancuronium bromide (2–4 μg ml^{-1}) added directly to the perfusate.

In situ experiments and measurements

Activities from phrenic nerve and whole hypoglossal nerve cut just before bifurcation of the lateral and medial branches were obtained via extracellular bipolar recordings using glass suction electrodes. To measure dose–response curve for ethanol, the phrenic nerve activity was recorded in eight of 15 control animals and paired recordings of both nerves were obtained in seven of 15 control animals and eight of eight ethanol-exposed animals. Another set of animals was used ($n = 4$ each for control and ethanol-exposed animals) for experiments concerning GABAergic neurotransmission. All nerve signals were amplified ($\times$ 50 000), filtered (0.3–3 kHz), integrated ($\tau = 50$–100 ms) and stored in a personal computer with the use of Spike 2 acquisition software (CED, Cambridge, UK). Experiments started after the amplitude and frequency of nerve recordings had stabilized with phrenic nerve activity displaying an augmenting ramp revealing a eupnoeic pattern (i.e. physiological) generated by the respiratory central network (St-John & Paton, 2003).

Ethanol was added to the perfusate in cumulative concentrations of 10, 20, 40 and 80 mM. For each concentration, measurements were performed 10 min after application, after nerve activity amplitude had stabilized. In some cases, small transient changes in the perfusion pressure were observed once ethanol reached the preparation and the perfusion pump rate was adapted accordingly. Our measurements were therefore performed at perfusion pressure values comparable to those during the predrug period. The respiratory cycle was defined according to phrenic nerve activity (Fig. 1*A*). Inspiration was defined as the duration of burst activity (Ti). Post-inspiration (PI), also called expiratory phase I, corresponded to decreasing postinspiration discharge after the end of inspiration and the second phase of expiration (TeII) was defined as the silence of phrenic nerve activity until the next burst. Expiration (Te) was the sum of PI and TeII durations. We also measured the preinspiration (Pre-I) phase duration on the hypoglossal nerve. The hypoglossal nerve also showed a postinspiration discharge (i.e. during the first part of expiration) with a longer duration than PI phase on the phrenic nerve. This was followed by a silence until the next pre-I burst. Then the equivalent period to phrenic expiration on hypoglossal nerve corresponds to the sum of hypoglossal postinspiratory discharge, the silence period and the Pre-I phase. Measurements of phase durations and inspiratory peak amplitude were performed on the integrated form of the recording. Respiratory frequency (R_f, cycles min^{-1}) was calculated from phrenic nerve

activity and we measured the time to maximal phrenic burst amplitude to analyse the pattern of inspiratory ramp discharge. To characterize GABAergic neurotransmission within the network in the two animal groups, we applied muscimol, a $GABA_A$ receptor agonist, directly into the perfusate at 50 μM (final concentration) and measured the time necessary to abolish phrenic nerve activity.

At the end of *in situ* experiments, animals were killed by a bolus of nembutal until complete arrest of the heart.

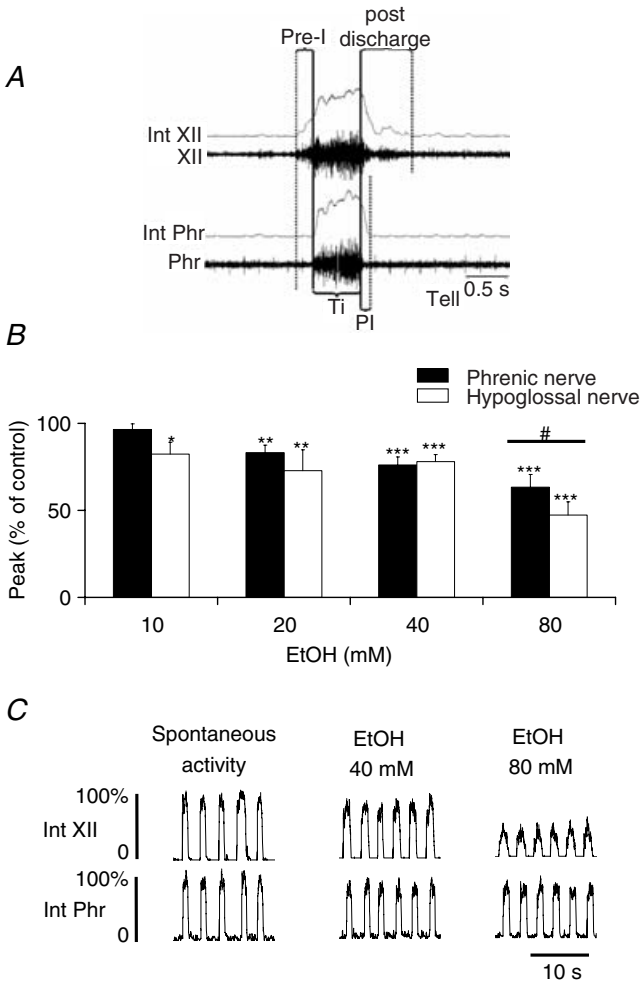

Figure 1. Acute ethanol exposure in control animals
A, illustration of respiratory phase duration measurements performed *in situ*. Pre-inspiration (Pre-I) phase, only present in hypoglossal (XII) nerve recording, corresponds to burst activity prior to onset of phrenic nerve (Phr) activity. Inspiration (Ti) is burst activity recorded on the phrenic nerve. Postinspiration (PI) duration was defined on the phrenic nerve, and corresponds to decreasing nerve activity after the end of Ti until silence is observed. Note that hypoglossal nerve also displayed a postinspiration discharge that was usually longer than the PI phase. The second part of expiration (TeII) is the period between the end of PI and the next inspiratory burst (not illustrated here). Expiration was the sum of phrenic PI and TeII durations. The equivalent period to phrenic expiration on hypoglossal nerve corresponds to the sum of hypoglossal postinspiratory discharge, the silence period and the Pre-I phase. *B*, ethanol effects on amplitude were significant from 10 mM for hypoglossal and 20 mM for the phrenic nerve. At 80 mM, a difference in sensitivity between the two nerves was observed (#$P < 0.05$). *C*, paired recordings of integrated phrenic (Int Phr) and hypoglossal (Int XII) nerves in the presence of 40 and 80 mM ethanol. The most intense effect was observed for 80 mM ethanol on hypoglossal nerve. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Recording breathing with plethysmography

Spontaneous respiratory activities in awake and unrestrained animals were measured by the barometric method (Bartlett & Tenney, 1970). Whole-body plethysmography was performed on five rats from each group (3 weeks old). After being weighed, animals were placed in a sealed Plexiglass chamber continuously flushed with humidified room air. This chamber was connected to a reference chamber of the same size and the pressure difference between the two chambers was measured with a high-gain differential pressure transducer (DP 45, Validyne). Animals were first allowed to acclimate to the chamber for a period of 30 min and measurements were performed for 90 s during states of immobility. Rectal temperature was measured before starting the experiments and after the respiratory measurements. Barometric chamber temperature was maintained above 31°C by an external heat source and was monitored throughout the experiment. Analogue signals were continuously digitized (micro 1401, CED electronics), recorded with software (Spike2, CED electronics) and stored on a personal computer for off-line analysis.

Data analysis

All results are presented as means ± S.E.M and expressed as percentage change from control values, unless otherwise stated. Measurements of *in situ* nerve activities were averaged on 15 consecutive respiratory cycles. Statistical analyses for ethanol concentration–response curves were performed on raw data with one- or two-way repeated-measures ANOVA, with nerves and drug treatment (ethanol *versus* water) as independent variables and nerve amplitude and phase duration as dependent variables. Pair-wise comparisons were performed with the Student–Neumann–Keuls *post hoc* test. The spontaneous rhythmic activity of the two nerves was compared between control and ethanol-exposed animals by Student's unpaired *t* test. We also determined whether chronic ethanol exposure affected animal body weight and body temperature between the two groups using Student's unpaired *t* test. $P < 0.05$ was considered significant.

For plethysmography recordings, respiratory phase durations (inspiration and expiration) as well as tidal volume (V_T) were measured and averaged on 30 consecutive respiratory cycles during immobility (quiet waking). Minute ventilation ($\dot{V}_E$) and R_f also were calculated. All comparisons between the two animal

groups were performed with Student's unpaired *t* test with a limit of significance of $P < 0.05$.

Results

Spontaneous activity and ethanol concentration–response curve in *in situ* preparations from control animals

Measurements of respiratory phases are illustrated in Fig. 1*A*. Before testing acute ethanol exposure, phrenic nerve mean frequency (R_f) was 17.3 ± 2.2 cycles min^{-1} with Ti and Te durations of 0.8 ± 0.06 s and 3.2 ± 0.29 s, respectively. PI and TeII durations were 0.25 ± 0.03 s and 2.9 ± 0.28 s, respectively. For hypoglossal nerve recording, discharge during PI and silent phase durations were 0.48 ± 0.17 s and 2.42 ± 0.52 s, respectively. In addition, pre-I discharge on hypoglossal nerve lasted 0.3 ± 0.05 s. Ethanol application induced a concentration-dependent decrease of amplitude on both nerves (Fig. 1*B*). Hypoglossal inspiratory burst amplitude decreased significantly at 10 mM ethanol ($-17.5 \pm 6.7\%$, $P < 0.05$), whereas phrenic nerve amplitude started to decrease at 20 mM ($-16.8 \pm 4.2\%$, $P < 0.01$). At 80 mM ethanol, hypoglossal nerve burst amplitude decreased by $52.8 \pm 7.8\%$ ($P < 0.001$) and phrenic nerve amplitude decreased by $36.8 \pm 7.5\%$ ($P < 0.001$), and a significant difference was observed between the response of these two nerves (Fig. 1*B* and *C*), demonstrating a higher sensitivity of the hypoglossal nerve to ethanol ($P < 0.05$) compared to the phrenic nerve.

Increasing the ethanol concentration did not affect inspiratory duration, but decreased the PI phase duration. PI phase duration for the phrenic nerve (Fig. 2*A*) was decreased by $10.0 \pm 2.9\%$ ($P < 0.05$) at 10 mM, and by $28.0 \pm 5.4\%$ at 80 mM ethanol ($P < 0.001$), whereas TeII phase duration was not significantly affected (Fig. 2*B*). Pre-I phase duration recorded on hypoglossal nerve was significantly increased only at 40 mM ethanol ($+69.2 \pm 20.8\%$, $P < 0.05$, Fig. 2*C*). Finally, R_f measured on phrenic nerve increased to reach a maximum of 23.3 ± 3.8 cycles min^{-1} at 80 mM ethanol ($P < 0.01$, Fig. 2*D*).

Spontaneous activity of respiratory nerves and breathing after chronic ethanol exposure

Animals used for *in situ* experiments after early chronic ethanol exposure did not show any significant difference in body weight compared to control animals (control, 91.5 ± 6.9 g; ethanol-treated group, 86.0 ± 5.2 g, $P > 0.05$). Inspiratory, PI and pre-I burst durations were not affected by chronic ethanol treatment. For example, phrenic burst duration was 0.8 ± 0.06 s for control and 0.79 ± 0.06 s for ethanol-treated animals (Fig. 3*A*). However, a significant 43% reduction in R_f was observed between the two populations (control, 17.3 ± 2.2 cycles min^{-1}; ethanol-exposed, 9.9 ± 1.2 cycles min^{-1}, $P < 0.05$). The decrease in R_f was due to a two-fold increase of Te duration on the phrenic nerve (control, 3.2 ± 0.29 s; ethanol-exposed, 5.9 ± 0.81 s, $P < 0.001$, Fig. 3*A*) and of the equivalent period of time on hypoglossal nerve recordings (control, 3.2 ± 0.46 s; ethanol-exposed, 5.9 ± 0.8 s, $P < 0.001$). The TeII phase was the most markedly affected, as it was increased by about 93% on phrenic nerve recordings (control, 2.9 ± 0.28 s ethanol-exposed, 5.6 ± 0.84 s, $P < 0.001$, Fig. 3*A*). Similarly, only the period of silence on hypoglossal nerve recordings was significantly increased by 125% (control, 2.4 ± 0.51 s; ethanol-exposed, 5.4 ± 0.85 s,

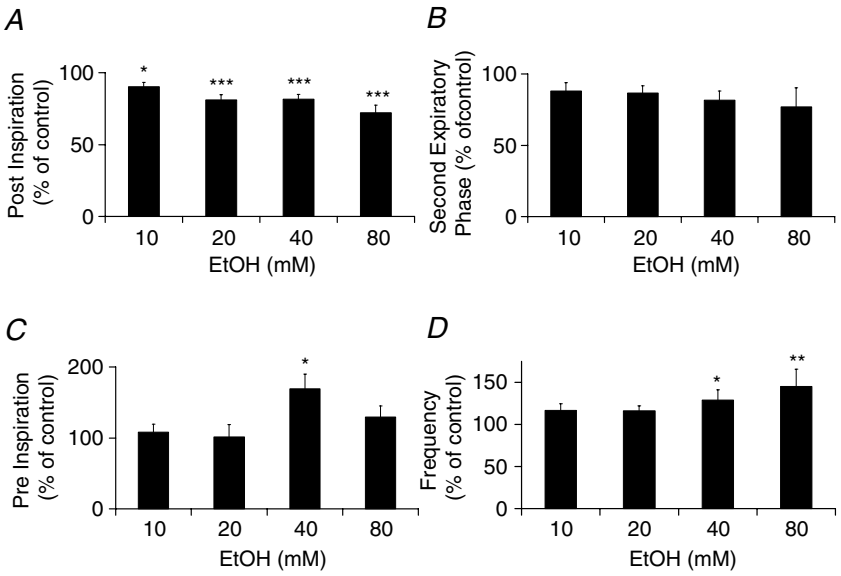

Figure 2. Acute ethanol exposure and respiratory phase durations in control animals studied *in situ*
A, phrenic postinspiration duration was reduced from 10 mM ethanol. *B*, the second expiratory phase on the phrenic nerve was not significantly reduced. *C*, on hypoglossal nerve, preinspiration burst duration (pre-I) increased but changes were significant only at 40 mM ethanol. *D*, respiratory frequency (R_f), as measured on phrenic nerve recording, increased with high doses of ethanol. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

$P < 0.001$). Furthermore, the slope of inspiratory ramp during phrenic nerve discharge (Fig. 3*B*) was significantly increased after chronic ethanol exposure (+31%, $P < 0.05$).

As a marked decrease in respiratory nerve frequency was observed in the *in situ* preparation, we determined whether this affected spontaneous breathing in intact and unanaesthetized animals by using plethysmography. Body weight and body temperature were not statistically different between ethanol-exposed and control animal groups ($n = 5$ for each). However, differences between control and ethanol-exposed animals were observed for spontaneous respiratory frequency (−23%, $P < 0.05$) and $\dot{V}_E$ (−39%, $P < 0.05$), whereas V_T was unchanged (see Table 1). Interestingly that the decrease in breathing frequency was due to a significant prolongation of expiration with no modification of the inspiratory duration (Fig. 4*A* and *B*) as seen in the *in situ* experiments. Furthermore, two control and two ethanol-exposed animals were tested in the *in situ* preparation 24 h after plethysmographic experiments. With plethysmography, the ethanol-exposed animals displayed about a 25% decrease in R_f compared to control animals. *In situ,* the respiratory frequency was decreased by about 40% in the same two ethanol-exposed animals compared to control animals (data not shown). Importantly, this series of experiments demonstrated that ethanol exposure early in life interferes with spontaneous breathing in 3- to 4-week-old animals.

Table 1. Effect of chronic and early ethanol exposure on body weight and ventilatory parameters in resting conditions

	Control group ($n = 5$)	Ethanol-exposed group ($n = 5$)
Body weight (g)	67 ± 9	64 ± 6
$\dot{V}_E$ (ml min^{-1} (100 g)$^{-1}$)	113 ± 9	69 ± 7*
R_f (cycles min^{-1})	149 ± 12	115 ± 4*
V_T (ml (100 g)$^{-1}$)	0.78 ± 0.08	0.62 ± 0.08

Data are expressed as means ± S.E.M. $\dot{V}_E$, minute ventilation; R_f, respiratory frequency; V_T, tidal volume. *Significant difference between the control group and the ethanol-exposed group ($P < 0.05$).

Ethanol concentration–response curve after chronic ethanol exposure

To test for tolerance or sensitization of the respiratory network to ethanol after early chronic ethanol exposure, we subjected ethanol-exposed animals to acute ethanol applications *in situ* (10–80 mM). Under these conditions, hypoglossal and phrenic nerve activities were found to be very sensitive to the depressant effects of ethanol, revealing a sensitization phenomenon. As illustrated in Fig. 5*A*, ethanol application after early chronic exposure to ethanol abolished much of the activity. This result was revealed with amplitude measurements. The amplitudes of the activities of the two nerves showed a dose-dependent response to acute ethanol (Fig. 5*B*) and difference in

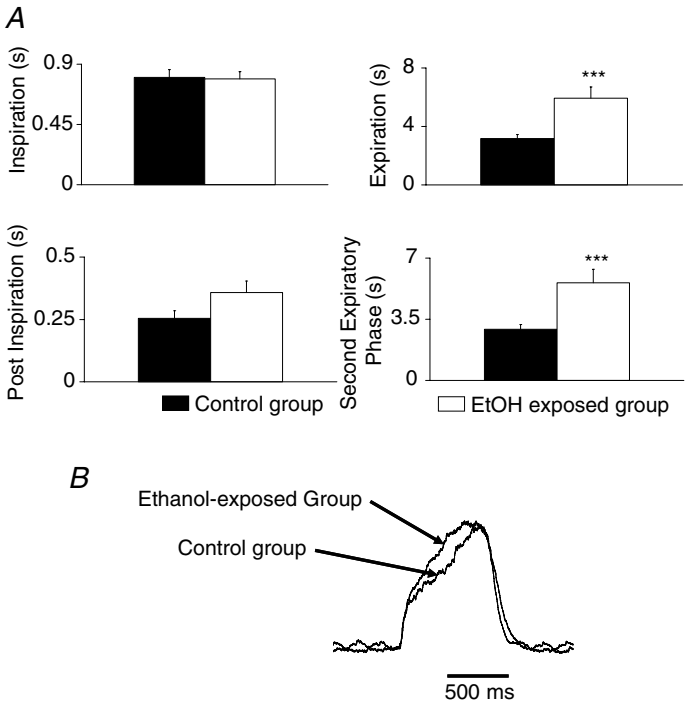

Figure 3. Early and chronic ethanol exposure alters spontaneous phrenic nerve activity *in situ*
A, no difference was observed in inspiratory duration. Expiration was increased (***$P < 0001$). During expiration, postinspiration was not significantly affected whereas the second expiratory phase was increased (***$P < 0001$). *B*, superimposed average of 15 consecutive phrenic nerve bursts from one control and one ethanol-exposed animal showing that the ramp became steeper in the ethanol-exposed animal whereas no difference was measured in inspiratory duration.

sensitivity between the two nerves was observed at a concentration of 10 mM ethanol instead of 80 mM in the control group ($P < 0.05$; cf. Figs 1*B* and 5*B*). In addition, in the control group, a response was still recorded in 100% of nerves at 80 mM ethanol, whereas in the chronic ethanol-exposed group, 37.5% of hypoglossal nerves were silenced at 10 mM and 100% were silenced at 80 mM ethanol. The phrenic nerve was also more markedly affected by ethanol application after chronic ethanol-exposure, as 12.5% of phrenic nerve recordings were silenced at 20 mM and 50% were silenced at 80 mM ethanol. Acute re-exposure to ethanol also induced an increase in frequency of phrenic nerve activity (see arrows in Fig. 5*A*) as observed in the control population, but this effect was not significant ($P > 0.05$). Because of the marked sensitivity in the two nerves to acute ethanol exposure, no statistical analysis was performed on phase durations.

Effects of $GABA_A$ agonist after early chronic ethanol exposure

The slower respiratory frequency measured *in situ* and *in vivo* after early and chronic ethanol exposure, suggested that ethanol exposure either increased inhibition within the respiratory network or decreased excitation. Indeed, ethanol has been shown to increase preferentially the inhibitory inputs onto hypoglossal motoneurones in slices (Sebe *et al.* 2003) and prenatal exposure to other drugs of abuse such as nicotine increased GABAergic inhibitions in the respiratory network *in vitro* (Luo *et al.* 2004). To test whether we had a similar situation, we measured the *in situ* effects of muscimol, a specific $GABA_A$ receptor agonist, in the two animal groups. In four control animal preparations (Fig. 6*A*), application of 50 μM muscimol directly into the perfusate abolished phrenic nerve activity after a mean delay of 52.8 ± 19.8 s. R_f before drug application was 18.3 ± 3.6 cycles min^{-1}. In two of these animals, washout of muscimol was performed by perfusing with drug-free aCSF, but no recovery was obtained after 30 min. Four ethanol-exposed animals were subjected to muscimol. In this group, control R_f was 7.6 ± 1.5 cycles min^{-1}. In two of the four ethanol-exposed animals tested, the powerful inhibitory effect of muscimol was not observed (Fig. 6*B*); that is, phrenic nerve burst activity persisted for more than 15 min in the presence of 50 μM muscimol without affecting either burst amplitude or frequency. Indeed, the muscimol concentration had to be doubled to abolish phrenic nerve activity in these two animals (data not shown). In the two other ethanol-exposed animals, phrenic nerve activity was abolished after a mean delay of 206.4 ± 29.1 s (+288% compared to control animals).

Discussion

Here, we show the consequences of ethanol exposure during the prenatal and postnatal periods on rhythmic respiratory nerve activity recorded from *in situ* juvenile rat preparations and on spontaneous breathing in intact and unanaesthetized animals. We also describe the sensitivity of respiratory nerve activity to acute ethanol application before and after chronic ethanol exposure, and to application of muscimol, a $GABA_A$ receptor agonist. The results show that (1) expiratory phase duration, measured both *in vivo* and *in situ*, is prolonged in ethanol-exposed animals, leading to a lower respiratory frequency, (2) re-exposure to ethanol in *in situ* preparations revealed a sensitization phenomenon on both the phrenic and hypoglossal nerves, after ethanol exposure during early life, and (3) rhythmic network activity measured *in situ* becomes tolerant to the inhibitory effect of a $GABA_A$ agonist after early exposure to ethanol.

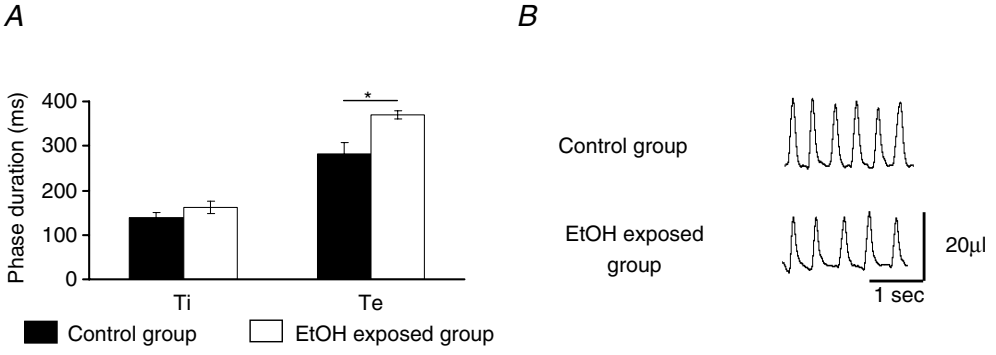

Figure 4. Respiratory activity under resting conditions recorded by whole-body plethysmography in intact and awake animals
A, respiratory phase durations in control animal (filled bars) and ethanol-exposed animals (open bars) showing that only expiratory duration was significantly increased after chronic ethanol exposure. *B*, examples of raw plethysmographic recordings from a control and an ethanol (EtOH)-exposed animal. Inspiration corresponds to the upward deflection of the trace and expiration to the downward deflection until the next upward deflection. Note that frequency was markedly reduced (−25%) whereas V_T was not significantly affected.

Consequences of chronic and early ethanol exposure on spontaneous rhythmic activity

This is the first time that the consequences of early chronic ethanol exposure on respiratory rhythmic activities have been investigated. Chronic ethanol exposure during fetal life and lactation in rats is equivalent to exposure during the second and third trimesters of pregnancy in humans (Dobbing & Sands, 1979; West, 1987). This type of exposure in humans induces fetal alcohol syndrome (Burd *et al.* 2003). In the present study, we have shown that similar exposure in rats significantly reduced the frequency of respiratory rhythmic activity derived from the brainstem in young rats. However, the reduction of spontaneous respiratory frequency after chronic ethanol exposure was more marked in *in situ* preparations than in *in vivo* experiments. This difference might be due to the basal frequency in the two experimental models. *In situ*, the control respiratory rhythmic activity rate was 20 cycles min^{-1} whereas *in vivo*, the respiratory rate in juvenile rats was as high as 150 cycles min^{-1}. These figures are in line with published results using the same models (St-John & Paton, 2000; St Jacques & St-John, 2000; Peyronnet *et al.* 2000). The *in situ* preparation lacks the Hering-Breuer reflex and is maintained at 31°C (this reflex and this temperature both slow spontaneous frequency), but a higher respiratory frequency can be achieved by increasing temperature or stimulating the vagus nerve (Paton, 1996; St-John & Paton, 2000; St Jacques & St-John, 2000).

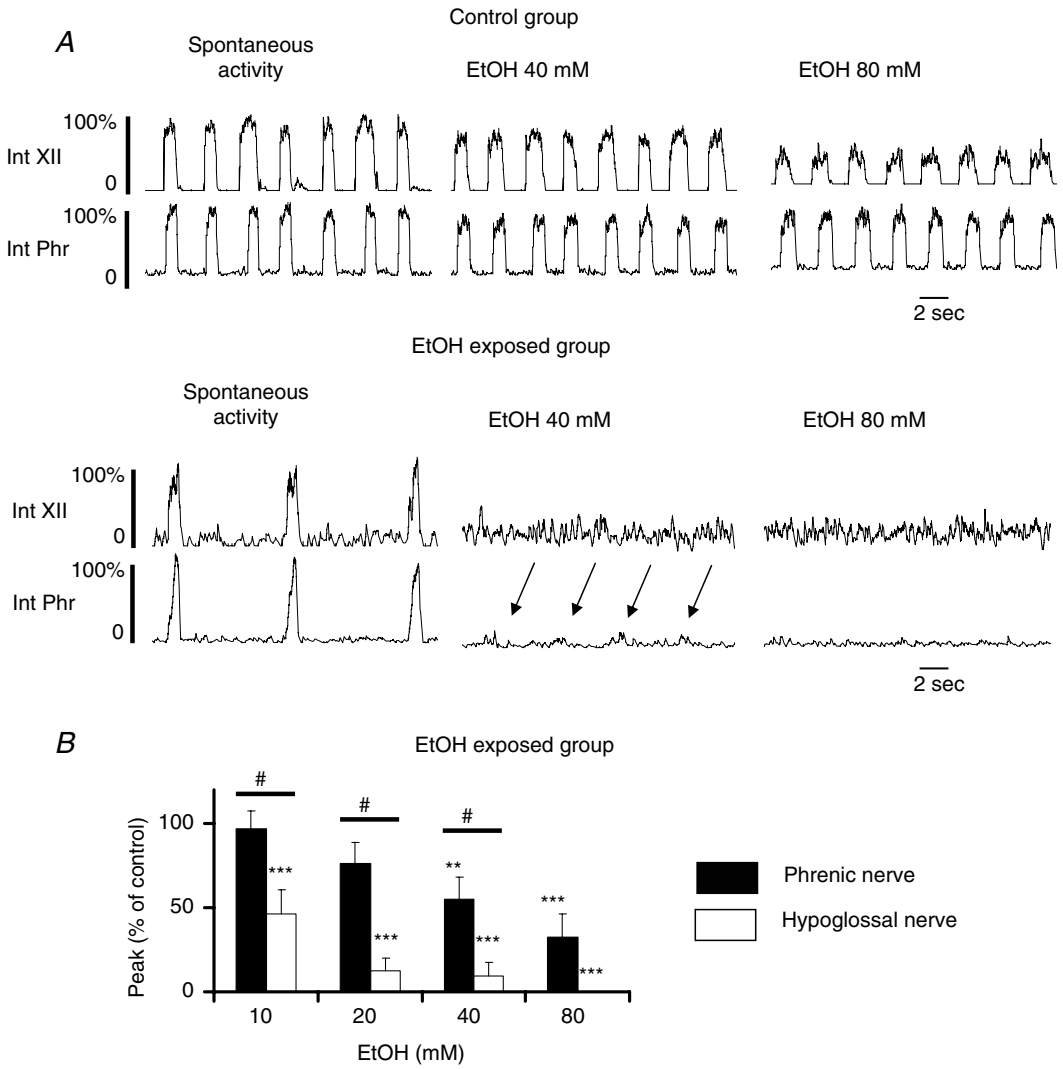

Figure 5. Ethanol concentration–response curve in ethanol-exposed animals studied *in situ*
A, paired recordings of phrenic (Phr) and hypoglossal (XII) nerve activity illustrated as integrated form (Int) showing effects of ethanol (40 and 80 mM) on control and ethanol-exposed animals. Rhythmic activity in ethanol (EtOH)-exposed animals is slower prior to ethanol application and is more sensitive to acute ethanol exposure. Arrows in the lower set of traces indicate the remaining phrenic nerve activity after application of 40 mM ethanol. No activity was observed on the hypoglossal nerve. *B*, effects of acute ethanol on amplitude in ethanol-exposed animals. Compare this with Fig. 1B. (***$P < 0.01$, dose-effect; #$P < 0.05$, response between nerves).

Phrenic inspiration and postinspiration durations were not affected by ethanol exposure in early life suggesting that off-switch mechanisms of inspiration were not altered. Meanwhile the incrementing ramp of phrenic burst increased, suggesting that inspiration developed more rapidly after chronic ethanol exposure. Interestingly only the expiratory phase duration was increased after chronic ethanol exposure in both *in situ* and *in vivo* experiments, leading to a reduced respiratory frequency. Furthermore, *in situ* experiments revealed that this effect was due to a specific increase in expiratory phase II duration. This alteration of the respiratory rhythm by ethanol is similar to that obtained *in vitro* with application of barbiturates or muscimol (Fregosi *et al.* 2004), or an increase in endogenous levels of GABA *in vivo* (Hedner *et al.* 1984). This suggests that chronic and early ethanol exposure results in a modulation of $GABA_A$-mediated inhibition within the network. We then hypothesized that an increase of GABA-mediated inhibitory transmission within the network would probably be at least in part responsible for the slower rhythm observed. This hypothesis has already been tested with respect to nicotine, another drug of abuse. Thus, prenatal exposure to nicotine induces an increase of GABA-mediated inhibition in the respiratory network (Luo *et al.* 2004). Moreover, it has been shown that chronic ethanol exposure *in utero* increased the number of muscimol binding sites in adult guinea-pig cerebral cortex (Bailey *et al.* 2001). However, application of muscimol in the present study revealed that the respiratory central network became less sensitive to this $GABA_A$ agonist, acting on the GABA site. Indeed, it shows that $GABA_A$ receptors became tolerant to exogenous GABA agonist after chronic ethanol exposure, as previously demonstrated. Kang *et al.* (1996) and Morrow *et al.* 1988), showed that muscimol-induced chloride uptake of synaptoneurosomes from rat cerebral cortex and chloride efflux in hippocampal slices were reduced after ethanol treatment. Muscimol is also less effective in inducing repetitive movements when injected into the substantia nigra after chronic ethanol exposure (Gonzalez & Czachura, 1989) and the GABA response of pyramidal cells from the rat piriform cortex or dissociated Purkinje neurones is reduced after chronic ethanol treatment (Hsiao *et al.* 1999; Signore & Yeh, 2000) with an increase in the EC_{50} of GABA (Signore & Yeh, 2000). The tolerance to muscimol reported after early chronic ethanol exposure, however, does not exclude the possibility that the effects of allosteric neuromodulators (such as neurosteroids; Allan *et al.* 1998; Ren & Greer, 2006) of the $GABA_A$ receptors have been increased to induce a lower respiratory frequency. Besides an effect of chronic ethanol exposure on the network inhibitory transmissions, we also cannot rule out the possibility that the excitatory transmissions have also been disturbed, as ethanol is known to reduce NMDA receptor activity (see below). However, further experimental data are needed to clarify this.

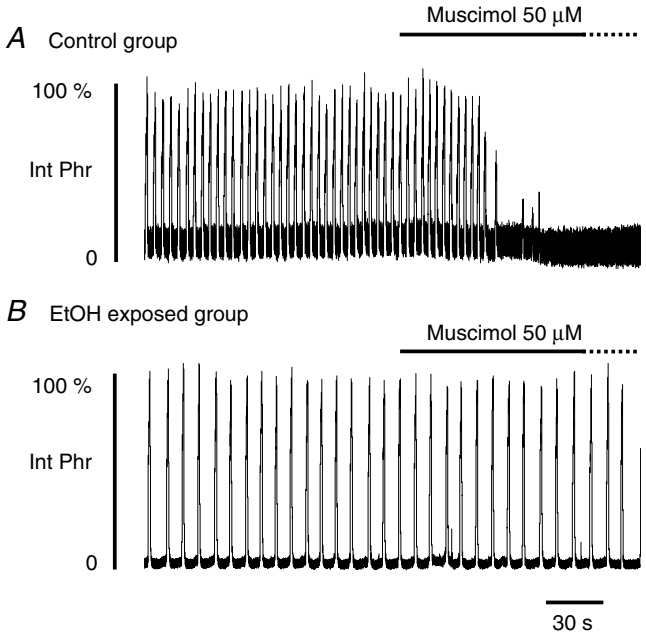

Figure 6. Effects of muscimol in control and ethanol-exposed animals

A, phrenic nerve activity in control animals *in situ* was rapidly abolished by application of 50 μM muscimol into the perfusate. *B*, in contrast to control animals, muscimol (50 μM) did not abolish phrenic nerve activity in two of four ethanol (EtOH)-exposed animals tested, whereas this activity disappeared in the two other animals after an interval three times longer than that observed in control animals.

Another interesting finding of our study was that chronic ethanol exposure induced opposite changes in respiratory frequency to those observed with acute application of ethanol. Whereas chronic early ethanol exposure reduced respiratory frequency with an increase in expiratory phase II duration, acute ethanol application increased respiratory frequency with a shortening of PI phase duration (see also Haji & Takeda, 1987; Takeda & Haji, 1990). Moreover, acute ethanol application strongly depressed inspiratory burst amplitude. These results suggest that different mechanisms are involved in the effects of chronic *versus* acute ethanol application, as previously shown by a biphasic effect of ethanol revealing neural adaptations after long-term ethanol exposure (for review see Faingold *et al.* 1998; Costa *et al.* 2000*b*). Acute ethanol exposure depresses respiratory activity probably because of its effects on NMDA, GABA and glycine receptors as demonstrated on hypoglossal motor outflow recorded from neonatal rat rhythmic slices (Gibson & Berger, 2000) or hypoglossal motoneuron recorded *in vitro* (Sebe *et al.* 2003). Acute exposure to ethanol also hyperpolarized about 50% of the respiratory neurons within the ventral respiratory group of the medulla of adult cats (Haji & Takeda, 1987; Takeda & Haji, 1990). After chronic early ethanol exposure, changes in respiratory frequency may reflect modifications of the central pattern

generator activity via cellular and/or neurochemical adaptive mechanisms which occur to compensate for the chronic presence of ethanol during brain development. However, these mechanisms are totally unknown at the present time and further investigations are needed to document the mechanism(s) underlying this effect.

Sensitivity to acute ethanol is increased after chronic ethanol exposure

Acute application of ethanol in control animals induced a depressive effect on the amplitude of the activity of the two nerves recorded with a higher sensitivity for the hypoglossal nerve compared to the phrenic nerve; this effect has not yet been explained. These results are in agreement with previous reports based on *in vivo* experiments in cats (Bonora *et al.* 1984; St John *et al.* 1986; Takeda & Haji, 1990). The phase duration changes induced by acute ethanol and the increase in frequency observed at relatively high ethanol concentrations were qualitatively similar to previous *in vivo* reports showing that the PI phase was the most sensitive (Haji & Takeda, 1987; Takeda & Haji, 1990). As the sensitivity of the *in situ* perfused rat preparation to acute ethanol is similar to that of *in vivo* preparations, it can be used to study the effects of chronic ethanol exposure on central network rhythmogenesis related to breathing function.

A striking observation in our study was the increase of depressant effects (sensitization) of acute ethanol on rhythmic network activity after early chronic ethanol exposure. The response of respiratory nerves to acute ethanol exposure was more marked after chronic ethanol exposure, as the nerves became silent with increasing ethanol concentrations; however, this effect was never observed in the control group. It is interesting that significant sensitization was obtained at 10 mM ethanol, a concentration almost equivalent to the legal maximum blood alcohol level permitted for vehicle driving in most European countries (typically $0.5\,g\,l^{-1}$). Sensitization of neuronal activity to acute ethanol after chronic exposure has already been reported in other areas of the brain (Brodie, 2002) and chronic ethanol exposure has also been shown to result in long-lasting behavioural sensitization (Abel & Berman, 1994). Our results strongly suggest that neurotransmitter systems underlying rhythmic activity within the network are sensitized by chronic ethanol exposure. The respiratory network activity is dependent on excitatory synaptic transmissions via glutamatergic receptors during burst activity and GABAergic/glycinergic inhibitory transmissions in the interburst interval (Bianchi *et al.* 1995). Acute ethanol exposure increases both types of inhibitory chloride currents, but reduces the NMDA-induced excitatory current. A higher sensitivity of both components to the effects of ethanol can at least partially explain our results. $GABA_A$ receptor sensitization is one of the possible mechanisms, as the more marked reduction in amplitude during acute ethanol exposure is likely to be due to ethanol-dependent sensitization of IPSP waves during inspiration. In this context, GABAergic synapses onto central dopaminergic neurons have been shown to be potentiated a long time after a single *in vivo* exposure to ethanol (Melis *et al.* 2002). *In utero* ethanol exposure has also been shown to alter GABAergic transmission. Accordingly, regulation of $GABA_A$ receptors by allosteric modulators in synaptoneurosomes from adult rat brain areas (Allan *et al.* 1998), GABAergic response of neocortical neurons in the offspring (Janiri *et al.* 1994) and neurons on central amygdala slices (Roberto *et al.* 2004*a*) have been shown to be altered by prenatal ethanol exposure.

However, our measurements performed with muscimol showed that phrenic burst was not modified by this $GABA_A$ agonist after early and chronic ethanol exposure. Although $GABA_A$ receptors became tolerant to this specific GABA activator, an increased sensitivity to positive modulators of $GABA_A$ receptors (benzodiazepines and neurosteroids; see Ren & Greer, 2006) after chronic ethanol exposure cannot be ruled out (Allan *et al.* 1998). Studies of NMDA receptor activity after early chronic ethanol exposure have shown that inhibition of the neuronal response to NMDA by acute ethanol is enhanced in the ventral tegmental area (Brodie, 2002) or central amygdala (Roberto *et al.* 2004*b*). In cultured Purkinje neurons, chronic ethanol exposure reduced neuronal responses to excitatory amino acids (Gruol, 1992). One of the possible mechanisms to explain the increased sensitivity to alcohol of burst amplitude after chronic exposure could therefore be sensitization of NMDA receptors to the inhibitory effects of acute alcohol. However, chronic ethanol exposure might also have affected the density of NMDA receptors, their subunit composition (Naassila & Daoust, 2002; Nixon *et al.* 2004) or their endogenous modulation (Costa *et al.* 2000*a*). Finally, sensitization to acute ethanol may involve another inhibitory neurotransmitter such as glycine, as acute ethanol preferentially enhances glycinergic inhibitory currents in hypoglossal motoneurons. This effect is more intense when measured in motoneurons during the juvenile period compared to the neonatal period, revealing a developmental aspect in the effects of acute ethanol exposure, probably linked to the maturational shift in receptor subunit composition (Eggers *et al.* 2000; Sebe *et al.* 2003). Because GABA and glycine are often co-released onto motoneurons, chronic ethanol exposure can modify both components of this inhibitory transmission. Furthermore, in addition to these neurotransmitters, chronic ethanol treatment may also affect dopaminergic or serotoninergic systems (Dizon *et al.* 2004; Sari & Zhou, 2004), which have been shown to play an important role in the respiratory network (Bianchi *et al.* 1995).

Physiological consequences of sensitization to ethanol

The slower rhythm observed *in situ* and *in vivo* in this study raises the question of chemosensitivity, which can be dramatically impaired in perinatally ethanol-exposed animals. In healthy humans, acute ethanol ingestion reduces the response to hypoxia (Series *et al.* 1990) and has a more marked depressant effect on genioglossal nerve output than on phrenic nerve output, resulting in reduced upper airway patency in the on-going presence of diaphragmatic effort (Krol *et al.* 1984). Other studies have reported that ethanol increases upper airway resistance in asymptomatic snorers (Mitler *et al.* 1988). In this context, sensitization to the depressant effect of ethanol on hypoglossal nerve activity after early chronic exposure can increase the incidence of obstructive sleep apnoea syndrome in humans, which is characterized by collapse of the upper airways during sleep. The lower $\dot{V}_E$ described in our study after early and chronic ethanol exposure revealed that these animals might be subjected to chronic hypoxia. In this case, peripheral (chemoreceptors) or central (respiratory ponto-bulbar network) specific adaptive mechanisms would be required to maintain viable breathing function.

Conclusion

Chronic ethanol exposure during the developmental period of the rat brain disturbs respiratory network activity, slowing its spontaneous frequency and reducing $\dot{V}_E$ in intact animals. Moreover, the rhythmic network became sensitized to the effects of acute ethanol application, suggesting sensitization of neurotransmitter systems within the network with glycine and glutamate being the most plausible candidates. Although a number of uncertainties persist regarding the mode of action of chronic ethanol exposure and the mechanisms involved in the development of sensitization, the present study demonstrates that early ethanol exposure alters the properties of the central rhythmic network and sensitizes its activity to the inhibitory effects of acute ethanol, which may lead to dramatic effects on the regulation of respiratory function.

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